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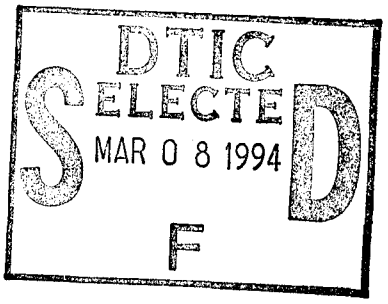

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13. ABSTRACT (Maximum 200 words)

Hepatitis E virus (HEV) infections represent a major health problem in developing countries. Presently, we have a limited understanding of the epidemiology and pathogenesis of the HEV. The goal of this phase II proposal was to develop diagnostic tests for HEV. Purification of recombinant HEV antigens augmented the development of serologic assays useful for large serosurveys. The prototype diagnostic test for HEV is convenient, sensitive and specific for anti-HEV. Our ELISA methodology detects both IgG and IgM specific anti-HEV and is therefore useful in diagnosing acute and past HEV infection. HEV infection can be verified by the presence of IgM antibody in the acute phase, seroconversion of IgG antibody in the convalescent phase, or by detection of HEV sequences in serum. With the recent development of serologic assays that distinguish acute and past hepatitis E infection, we are currently able to: 1) measure the worldwide prevalence of the virus, 2) access its endemicity, 3) determine the duration of antibody, and 4) engage in disease association studies to correlate antibody response with immunity.

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FOREWORD

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Patrice O. Yost 1/10/95

PI - Signature Date

I. INTRODUCTION

Hepatitis E virus (HEV) is the major etiologic agent causing enterically-transmitted non-A, non-B hepatitis (ET-NANBH, Purcell and Ticehurst, 1988, Bradley et al, 1988). Epidemics have been reported in Asia, Soviet Union, India, Africa, Mexico, and Central America (Bradley et al, 1988, Purcell and Ticehurst, 1988). Contamination of drinking water with raw sewage is thought to be the principle vehicle of virus transmission. Ticehurst (1991) has proposed that the virus enters the blood via the gastrointestinal tract, replicates in the liver, is released from hepatocytes to bile and blood and then excreted in feces. Feces obtained during the course of infection contain 29-32nm virus like particles (VLPs, Bradley et.al., 1988).

HEV infections represent a major health problem in developing countries. The disease affects mostly young to middle age adults with an overall mortality rate of 1%. However the highest case fatality rates (10-20%) occurs in pregnant women who develop fulminant hepatitis (Purcell and Ticehurst, 1988). This phenomena may be due to viral pathogenicity or to the interaction of the virus with an immunosuppressed pregnant host. Presently, we have a limited understanding of the epidemiology and pathogenesis of the HEV.

The molecular characterization and sequencing of the Burma strain HEV viral genome (Reyes et.al., 1990, Tam et al, 1991) established HEV as a unique positive sense, single stranded, polyadenylated RNA virus. Sequence data and the genomic organization of HEV distinguishes it from HAV, HBV, HCV and HDV. Although, it has been proposed that HEV may belong to the calicivirus family because of its size, sedimentation coefficient and sensitivity to CsCl, no significant sequence homologies were found between HEV and feline calicivirus (Tam et.al., 1991). Notably, HEV amino acid patterns place the virus in the 'alpha-like' superfamily. Comparative analysis of conserved function domains show that HEV is more similar to alphavirus, furovirus and rubella virus (Purdy et. al., 1993). Certain features suggest that HEV is related to both the enveloped alphaviruses and the non-enveloped caliciviruses. The final taxonomic classification of HEV remains to be determined.

Cloning of HEV led to the identification of type-common structural epitopes. The isolation of two immunodominant epitopes from the HEV Mexico and Burma strains (Yarbough et.al., 1991) and their cross-reactivity with human, chimp and cyno sera indicated that these epitopes would have utility in the diagnosis of HEV infections. During the funding of the phase I proposal, we characterized the two immunodominant epitopes encoded by ORF2 and ORF3 and demonstrated the utility of the cloned antigens to detect antibody to HEV in serum specimens. However since the lambda gt11 phage plaque assay we employed was laborious, we persisted to develop a rapid and simple test for screening large numbers of sera for antibody to HEV. Purification of recombinant HEV antigens led to the development of practical serologic assays

for anti-HEV useful for large serosurveys.

The goals of this phase II proposal was to develop diagnostic tests for HEV antibodies based on enzyme-linked immunoassays. To augment diagnosis of hepatitis E virus infection, we proposed to develop confirmatory test to detect HEV nucleic acid in human fecal and serum specimens. The expected benefits of the research was to develop a convenient and sensitive antibody assay to diagnose acute HEV infection and to determine seroprevalence of HEV in various populations groups.

II. METHODOLOGY AND RESULTS

Technical Objective 1

Our goal was to express HEV proteins from open reading frames (ORF) 1, 2, and 3, and purify to homogeneity to use as antigens in ELISA. For protein expression in E. coli, HEV sequences were cloned into the pGEX vector system as fusions with the C terminus of glutathione-S-transferase (GST) from Schistosoma japonicum (Smith and Johnson, 1988). ORF1 (see figure 1) from HEV Burma was cloned in the pGEX fusion system. The construct contained the unique Nde1 site 17 bp upstream of the first in frame ATG of ORF1 and continued through basepair 5679 at the unique Hind3 site, 570 basepairs downstream of the stop codon. Western blot analysis, using acute and convalescent phase human sera did not identify any protein products expressed in E. coli. We propose to develop a strategy to clone and express these non-structural regions in baculovirus and vaccinia.

Expression of a small antigenic determinant, 4-2, located at the carboxyl terminus of ORF3 (see figure 1) had previously been show to be useful for binding anti-HEV specific antibodies. We expressed the full length ORF3 of 123 amino acids as GST fusions in E. coli to determine if there were additional antigenic determinants in ORF3 not represented in the 33 amino acid epitope of 4-2. Unlike the smaller epitopes of ORF3, the full-length fusion was expressed as an insoluble protein in inclusion bodies.

Expression of a small antigenic determinant, 3-2, located at the carboxyl terminus of ORF2 (see figure 1) had previously been show to be useful for binding anti-HEV specific antibodies. In an effort to express an ORF2 protein with improved anti-HEV detection, we cloned and expressed as a GST fusion protein in E. coli the 327 amino acids (clone designation, SG3) from the 3' end of putative capsid gene (ORF2) in HEV. Since the SG3 was expressed as an insoluble protein in E. coli and was difficult to purify, the protein was expressed with a histidine tail at its 3' end. This permitted purification by immobilized metal affinity chromatography (IMAC).

Proteins expressed in baculovirus are known to preserve the biological properties of many proteins. It was expected that expression of the complete ORF2 of HEV would result in a capsid

protein that might self-assemble into HEV virus-like particles. Expression of ORF2 in Sf9 cells using recombinant baculovirus yielded a 73 KD protein. Protein levels were 10-30% of total cell mass. Although expressed as an insoluble protein, we have refolded the 73 KD ORF2 protein and used it to coat ELISA plates.

The 73K ORF2 protein expressed in insect cells seemed to undergo a proteolytic cleavage to yield a smaller soluble protein species of approximate 62 KD. We presume this 'cleavage' to be mediated by a baculovirus-specific proteinase. This cleaved form was purified to homogeneity and applied to a S-1000 molecular sizing column to evaluate protein conformation. The 62K ORF2 behaved as a particulate material. To evaluate the antigenicity of the purified protein, immunoassays were performed.

Technical Objective 2

The principles of the HEV ELISA are shown in figure 2. Recombinant HEV antigens were diluted in 0.1 M carbonate buffer pH 9.5 and coated onto each well of a polystyrene microtiter plate at 200 ng/100 ul. After overnight incubation, the wells were washed in phosphate-buffered-saline-Tween (PBS-0.05% Tween) and blocked with antibody diluent containing bovine serum albumin (BSA), goat serum, gelatin, and powdered milk. Serum samples diluted at least 1:100 in antibody incubation buffer were added to incubate for 1 hour at room temperature or 30 minutes at 37°C. The wells were washed to remove any unbound antibody and incubated with horseradish peroxidase conjugated goat anti-human IgG (gamma chain specific) or IgM (mu chain specific). After a final wash, substrate is added at room temperature for 20 minutes. The plates were read at an absorbance of 490 nm. A serum positive for anti-HEV has an ELISA signal greater than 3-fold the signal generated with non-recombinant GST and value above the cutoff of 0.190 for non-human primate sera and 0.350 for human sera. The specificity of the HEV ELISA was demonstrated by the lack of significant cross-reactivity with specimens from other hepatitis disease categories (figure 3).

Screening a panel of acute viral hepatitis cases (figure 4) verified that all the significant antigenic determinant(s) of ORF3 were contained within the small 4-2 epitope. Since the larger full-length ORF3 was expressed as an insoluble protein, we disregarded its use as a diagnostic tool and declared the 4-2 epitope as representative of the ORF3 region from HEV strains, Mexico (M) and Burma (B).

By testing various sera from acute hepatitis E panels by ELISA, we reported greater seropositivity with the SG3 antigen. One representative panel is a panel of acute hepatitis cases from the Abbottabad, Pakistan outbreak (figure 5). In this panel the SG3 antigen detected additionally 27% and 34% IgG and IgM anti-HEV in sera specimens that were previously undetected by the smaller ORF2 and ORF3 epitopes. This data was supportive evidence for at least one additional epitope in ORF2 that lie outside the previously identified 3-2 region encoded by the 42 amino acids at the 3' end of ORF2. We had demonstrated the

utility of the ORF2 antigens to detect IgM specific anti-HEV and to discriminate between acute and convalescent phase anti-HEV.

Since proteins expressed in baculovirus are known to preserve the antigenic and immunogenic determinants of proteins, we investigated improving our anti-HEV detection by using proteins expressed in insect cells. In our initial immunoassays, we observed that the full-length 73K ORF2 did not seem to contribute an improvement over the E. coli based HEV ELISA. Further investigation showed that the proficiency of the 73K protein to bind antibody in solution was related to the temperature that the purified protein was stored. 73K protein was sensitive to freeze thawing. If, however, stored at 4°C, the 73K ORF2 detected anti-HEV in serum samples as well as the previously purified E. coli proteins (figure 6). The soluble 62K ORF2 species that has properties that resemble native virions, is a very stable protein. The protein is stores well at both 4°C and -20°C, and resists degradation with freeze-thaw cycles.

By comparing the antigenicity of the SG3, 73K and 62K ORF2 proteins in pedigreed panels, we observed improved anti-HEV detection with the processed form of 62K (figure 6). Using 62K, 165 and 39% more sera were scored as IgG and IgM anti-HEV positive. The results demonstrated that the sensitivity and specificity of the assay was significantly improved using the 62K as an antigen source. The results suggest that the 62K protein expressed in baculovirus may retain conformational structures that resemble the native virion.

Technical Objective 3

Polyclonal rabbit antisera to HEV antigens from ORF3 and ORF2 have been raised (figure 7). These sera have been useful in ELISA and western blot analysis to evaluate specific antibody reactivity. We recently immunized two rabbits with the purified 73K ORF2 protein expressed in baculovirus.

We used purified IgGs specific to the immunodominant epitopes at the 3' end of ORF2 to identify which rabbit antibodies would bind HEV VLPs. We observed a significant difference in the ability of these rabbit polyclonal antibodies directed to HEV recombinant proteins to bind HEV antigen in the liver of infected cynos. Compared to human IgGs from a naturally infected individual, rabbit IgGs bind HEV antigen poorly. This may be supportive evidence for the generation of antibodies to conformational epitopes upon natural infection.

Our initial attempt to generate a panel of SG3 monoclonals was hampered by the highly immunogenic thrombin cleavage site contained within the SG3/GST fusion protein used to immunize the mice. In a subsequent attempt, mice were immunized with a SG3/GST fusion protein lacking the thrombin cleavage site. We identified 14 monoclonal antibodies directed to SG3 (figure 7). All except two were IgM isotype. One monoclonal mapped to the immunodominant 3-2 region at the 3' end of ORF2. Overall, the avidity of the monoclonals to bind HEV protein is poor, as

demonstrated by ELISA titering.

We have since initiated another protocol to generate monoclonals to the purified 62K ORF2 protein. Fusions are complete and screening of subclones are in progress. These antibodies will be used to map neutralizing antibodies to HEV.

Technical Objective 4

We developed a confirmatory test for detecting HEV based on polymerase chain reactions to detect specific nucleotide sequences of HEV. Sequences from the RNA-directed RNA polymerase regions within ORF1 (figure 8) were previously shown to be useful for HEV detection. However, the greater sequence homology within ORF2 between divergent HEV strains made ORF2 sequences ideal candidates for further investigation. We evaluated seven primer sets that correspond to overlapping regions of HEV ORF2 for PCR amplification of HEV viral RNA (figure 8,9). For broad detection, the most useful primers were those which mapped to the 3' end of the highly conserved region of ORF2. We have shared these primer sets with investigators at WRAIR; the primers have been useful in amplifying HEV sequences from various Asian and Mexico like strains of HEV.

We have been able to detect HEV sequences in human sera from persons with acute hepatitis E infections. Sera from children with acute hepatitis in Egypt were tested by RT-PCR for HEV (figure 9). Two of three anti-HEV positive samples were confirmed to contain HEV sequences, thereby confirming acute HEV infection.

Technical Objective 5

It has been long speculated that HEV causes acute sporadic hepatitis in regions where HEV is endemic. The recently developed diagnostic tests for anti-HEV has confirmed on-going exposure to HEV in endemic regions. In developing countries, 7-16% of the population have measurable antibodies to HEV (figure 10).

In developed countries, HEV antibody tests suggest that 1-3% of the population have been exposed to virus (figure 10). The prevalence of HEV antibody in asymptomatic and symptomatic persons have not been rigorously compared, however all of the anti-HEV positive sera from U.S. blood donors were from persons without signs of clinical hepatitis. There is a strong association between anti-HEV in donors and endemic regions of the virus (figure 11). None of the anti-HEV positive sera were confirmed acute cases by IgM analysis. Nor was there any indication of viremia. This suggest that acquisition was not in the U.S.

To date we have not confirmed any chronic hepatitis E infections. Acute and fulminant hepatitis E cases in developed

countries have been confirmed by antibody test and PCR. However, all confirmed fulminant cases associated with HEV were from persons who lived or travelled to areas endemic for HEV. To evaluate the risk for exposure to HEV in U.S. travellers, we tested patients seen in travel clinic before travelling, six weeks after travelling, and six months after travelling (figure 12). Travel destinations were diverse. The presence of antibody in persons with prior travel history support the observations that HEV antibody can be detected for several years. Seroconversion in a few individuals (3%) are consistent with exposure to virus while travelling. Since none of the subjects reported any symptoms of hepatitis before or after travel, we infer subclinical infections.

III. CONCLUSIONS

The prototype diagnostic test for HEV is convenient, sensitive and specific for anti-HEV and enables the identification of specific anti-HEVs in as little as 1 ul of sera. The HEV ELISA has been tested for cross-reactivity with serum antibodies to HAV, HBV, HCV, Norwalk and Astrovirus and shown to be specific for anti-HEV. The specificity of the ELISA using recombinant antigens is 97.5% as shown by specimens obtained from a non-endemic region for hepatitis E infection. Our ELISA methodology detects both IgG and IgM specific anti-HEV and is therefore useful in diagnosing acute and past HEV infection. HEV infection can be authenticated by the presence of IgM antibody in acute phase, seroconversion of IgG antibody in convalescent phase, or by detection of HEV sequences in serum by RT-PCR.

Testing for hepatitis E is not routinely performed in diagnostic laboratories. With the recent development of serologic assays that distinguish acute and past hepatitis E infection, we are currently able to: 1) measure the worldwide prevalence of the virus, 2) access its endemicity, 3) determine the duration of antibody, and 4) engage in disease association studies to correlate antibody response with immunity.

A vaccine to HEV could prevent epidemics and acute sporadic cases of hepatitis E in developing countries and to persons travelling to these regions. To be effective in the development of an HEV vaccine, it will be necessary to establish a standard assay to be used by investigators to evaluate the mechanism of hepatitis E virus neutralization.

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Purdy, M.A., Tam, A.W., Huang, C.C., Yarbough, P.O., and Reyes, G.R. 1993. Hepatitis E Virus: A Non-Enveloped Member of the 'Alpha-Like' RNA Virus Supergroup? Seminars in Virology 4:319-326.

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Yarbough, P. O., et al. 1991. Hepatitis E Virus: Identification of type-common epitopes. J. Virol., 65:5790-5797.

V. APPENDIX

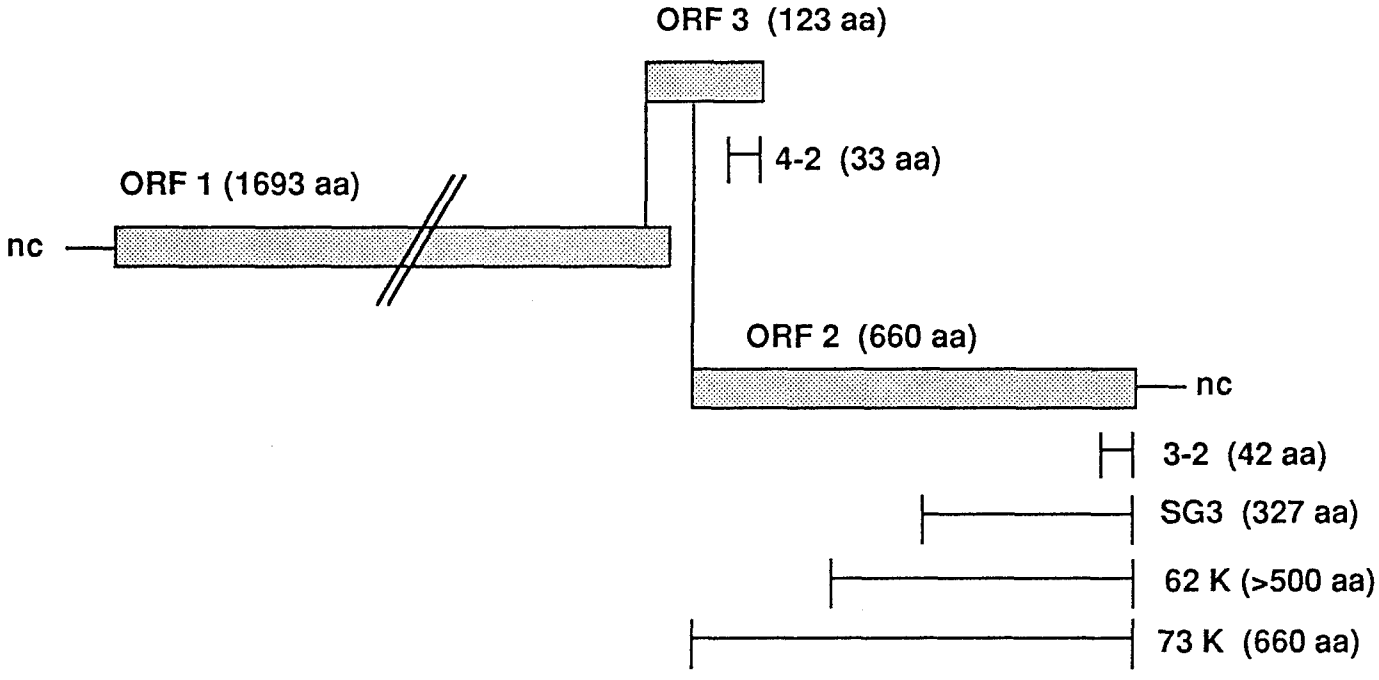
Figures

Reprints

Yarbough, P.O., Tam, A.W., Gabor, K., Garza, E., Moeckli, R.A., Palings, I., Simonsen, C., and Reyes, G.R. 1994. Assay Development of Diagnostic Tests for Hepatitis E. In: Viral Hepatitis and Liver Disease, eds. K. Nishioka, H. Suzuki, S. Mishiro, and T. Oda, Springer-Verlag Tokyo, pp. 367-370.

HEPATITIS E VIRUS GENOME

LOCALIZATION OF IMMUNOREACTIVE EPITOPES



- ORF1 is believed to encode the viral non-structural proteins including the RNA-dependent RNA polymerase. ORF2 is believed to encode the putative capsid structural gene. The function of ORF3 is undetermined.

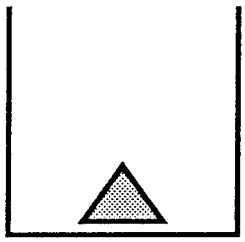
- Immunoreactive epitopes have been identified in both ORF2 and ORF3. HEV clones 4-2 and 3-2 were isolated from a lambda gt11 cDNA library constructed from an extract of a human stool sample. SG3 was generated by PCR amplification using sequence-specific primers.

- Each of 4-2, 3-2, and SG3 were expressed as GST fusion proteins in E. coli. Expression of ORF2 in Sf9 cells using recombinant baculovirus yielded a 73 kDa protein (73 K). Subsequent cleavage of 73 K by a baculovirus-specific proteinases produced a soluble 62 kDa protein species (62 K).

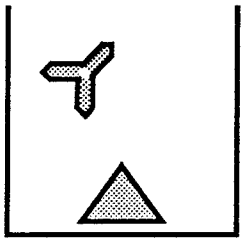
- These viral protein-encoding regions were used to develop an ELISA diagnostic assay to detect antibody to HEV. The ELISA detects both IgG and IgM specific anti-HEV and is useful in diagnosing acute and past HEV infection.

PRINCIPLES OF THE HEV ELISA

1. HEV antigen-coated well. (200 ng)

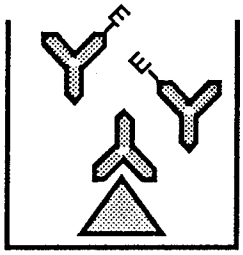


2. Add serum or plasma at 1:100 dilution. Incubate 30 minutes at 37°C.

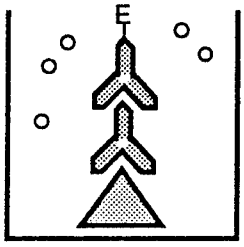


3. Wash / aspirate unbound material.

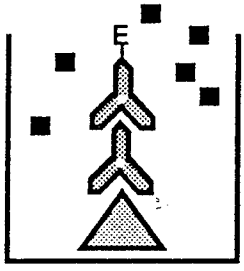
4. Add horseradish peroxidase conjugate. Incubate at 37°C.



5. Wash / aspirate excess conjugate.



6. Add substrate. Incubate 15 minutes at room temperature.



7. Add stop solution.

8. Read absorbance at 492 nm.

HEV antigen

HEV antibodies

Horseradish peroxidase conjugate

substrate

Yellow-orange color

ANTI-HEV IN VARIOUS DISEASE CATEGORIES

Disease Category	Anti-HEV		
	No.	IgG	IgM
Acute hepatitis A	34	3	3
Convalescent hepatitis A	5	0	0
Acute hepatitis B	10	1	1
Convalescent hepatitis B	10	0	0
Anti-HBc reactive	10	0	1
Acute NANB	19	0	0
Rheumatoid factor[1]	20	2	0
ANA reactive	14	0	0
Acute rubella	5	0	0
Convalescent Norwalk virus[2]	4	0	0

[1] 10-2000 IU/ml.
[2] Chimpanzee (Wyatt et al., 1978)

Figure 3

HEV ANTIGEN SPECIFICITY
Acute Viral Hepatitis Cases

IgG SCREENING		
ORF2	**SG3 (B)**	**97 positive**
	3-2 (M)	**74**
	SG3 (B)/3-2 (M)	**68**
ORF3	**4-2 (M)**	**48 positive**
	4-2 (B)	**53**
	8-5 (B)	**52**
	All antigens	**40 positive**
	SG3 (B) *only*	**29 positive**
	3-2 (M) *only*	**6**
	4-2 (B) *only*	**1**
	4-2 (M) *only*	**1**
IgM SCREENING		
	SG3 (B)	**38 positive**
	3-2 (M)	**26**
	4-2 (B)	**6**
	8-5 (B)	**3**
	4-2 (M)	**2**

Figure 4

ANTI-HEV DETECTION WITH SG3

(Abbottabad, Pakistan Outbreak)

Anti-HEV IgG

	# Tested	Antibody to SG3 (B)	3-2 (M)	4-2 (B)	4-2 (M)
Acute	26	24 (92%)	17 (65%)	13 (50%)	7 (27%)
Early conv	17	16 (94%)	10 (59%)	12 (71%)	4 (24%)
Conv	18	16 (89%)	4 (22%)	1 (6%)	0

Anti-HEV IgM

	# Tested	Antibody to SG3 (B)	3-2 (M)	4-2 (B)	4-2 (M)
Acute	26	11 (42%)	2 (8%)	0	0
Early conv	18	8 (44%)	0	0	0
Conv	19	0	0	0	0

IMPROVED DETECTION OF ANTI-HEV

BACULOVIRUS EXPRESSED PROTEINS

ANTI-HEV IgG

	Antibody to		
	62 K	SG3	73 K
Borneo S89	2.579	0.895	1.077
FVH 3	1.298	0.338	0.374
FVH 11	2.416	1.237	1.247
FVH 26	1.353	0.397	0.571
FVH 29	1.051	0.515	0.540
FVH 31	2.474	0.401	0.494
MB0283	2.771	2.602	2.558
MB0288	2.506	2.088	2.311
Som 002	2.616	1.357	1.301
Som 010	2.617	2.489	2.588
Som 032	2.503	2.456	2.351
Som 055	2.386	1.930	1.539
* Som 428	1.687	0.284	0.312
* Som 443	0.727	0.246	0.337
* Som 458	2.441	0.206	0.257
Som Pool #3	2.474	2.305	2.235
Sudan 54	2.717	2.159	2.310
Sudan 60	2.541	2.264	2.402

ANTI-HEV IgM

	Antibody to		
	62 K	SG3	73 K
* Borneo S89	2.527	0.237	0.356
FVH 3	0.164	0.064	0.061
* FVH 11	0.405	0.091	0.115
* FVH 26	0.319	0.077	0.069
* FVH 29	0.493	0.149	0.115
FVH 31	0.223	0.080	0.061
* MB0283	0.892	0.123	0.147
* MB0288	0.335	0.083	0.090
Som 002	2.598	0.508	0.681
Som 010	2.526	0.534	0.939
Som 032	2.861	0.722	0.877
Som 055	2.560	1.342	1.283
Som 428	2.463	1.165	0.863
Som 443	1.106	0.411	0.392
* Som 458	1.525	0.279	0.248
Som Pool #3	2.379	0.942	1.428
Sudan 54	2.652	0.532	0.754
Sudan 60	0.529	0.396	0.414

ANTIBODIES DIRECTED TO HEV EPITOPES

ANTIBODIES	IMMUNOGEN	ISOTYPE	LOCALIZATION
Murine Monoclonals			
BT 9, 24-27, 30-36	SG3 (B)	IgM	ORF2
BT 28	SG3 (B)	IgG_2	ORF2
BT 29	SG3 (B)	IgG_1	ORF2
Rabbit Polyclonals			
22	4-2 (B)	poly	ORF3
30	4-2 (M)	poly	ORF3
J1055	ORF3 (B)	poly	ORF3
23	3-2 (B)	poly	ORF2
J1019	SG3 (B)	poly	ORF2

Figure 7

AMPLIFICATION OF HEV VIRAL RNA

NAME		SEQUENCE	POSITION
ORF1			
ET1.1	F1:	5' GCT(C)ATTATGGAG(A)GAGTGTGG 3'	4461
	R1:	5' CAGGG(C)CCCCAA(G)TTCTTCTC 3'	4878
	F2:	5' GCGTGGATCT(C)TGCAGGCC 3'	4524
	R2:	5' TTCAACTTCAAG(A)CCACAGCC 3'	4762
ORF2			
3.2	F1	5' GCCGAGTATGACCAGTCCAC 3'	6581
	R1	5' ACAACTCCCGAGTTTTACCC 3'	7130
	F2	5' AATGTTGCGACCGGCGCGC 3'	6653
	R2	5' TAAGGCGCTGAAGCTAGCG 3'	7100

Figure 8

Figure 9

AMPLIFICATION OF HEV

PRIMER SELECTION FOR ORF1, ORF2 REGIONS of HEV

India pool sera
Egypt #36C
Egypt #3B
Egypt #2B
Egypt #1B
India #54
Normal sera
India pool sera

ORF2 7 F&R
6 F&R
5 F&R
4 F&R
3 F&R
2 F&R
1 F&R
ET 1.1 F2,R2
F1, R1

PREVALENCE OF ANTI-HEV

SAMPLES	#SERA	#POSITIVE(%)		COUNTRY
Blood donors	1,000	23	(2.3%)	U.S./California
	268	9	(3.4%)	U.S./California
	168	2	(1.2%)	U.S./Texas
Blood donors	66	6	(9.1%)	Mexico
Random illness	100	0	(0.0%)	Germany
Random donors	275	5	(1.8%)	Netherlands
Random donors	1,275	14	(1.1%)	Netherlands
Random healthy	1,367	76	(5.5%)	Turkey
Random healthy	374	18	(4.8%)	Sudan
Random donors	189	5	(2.6%)	Japan
Random donors	470	46	(9.8%)	Korea
Random healthy	100	15	(15.0%)	Thailand
Random healthy	87	3	(3.4%)	Singapore
Blood donors	360	14	(3.9%)	Singapore
Random healthy	355	57	(16.1%)	Hong Kong
Random donors	551	54	(9.8%)	China
Random donors	305	22	(7.2%)	China
Random >20 yrs	384	41	(10.7%)	Taiwan
Random <20 yrs	600	2	(0.3%)	Taiwan
Random donors	279	1	(0.4%)	Australia

ANTI-HEV IN USA
(ASSOCIATION WITH ENDEMIC / EPIDEMIC REGION)

DONOR PLACE OF BIRTH	IgG anti-HEV +	IgM anti-HEV +	HEV RNA PCR +
Born in endemic areas n = 97	11 (11%)	0	0
US Born: Both parents born in endemic areas n = 62	6 (10%)	0	0
US Born: One parent born in endemic areas n = 36	1 (3%)	0	0
US Born: Treated Hemophiliacs n = 276	9 (3%)	0	0
US Born: Blood donors n = 90	2 (2%)	0	0

RISK OF HEPATITIS E in US TRAVELLERS

Anti HEV IgG

Pre-Travel	Post-Travel
5/250 (2%)	13/250 (5%)

Areas of Travel:	Number of Patients:
Asia	128
Middle East	11
Africa	13
Central America	14
South America	13
Others	3

Risk Factors:	Number of Patients (%):
Uncooked food consumption	42
Unboiled (or unbottled) water consumptio	32
Exposure to icteric subject	0

Symptoms After Travel:	Number of Patients (%):
Nausea or Vomiting	15
Diarrhea	43
Abdominal Pain	13
Fever	5
Jaundice	0

Figure 12

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Assay Development of Diagnostic Tests for Hepatitis E

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The development of convenient, sensitive, and specific diagnostic tests to investigate the serology of hepatitis E virus (HEV) infection will be crucial to the understanding of the immune response to this virus. Previously, we reported the identification of two cDNA clones from the 3′ end of open reading frame (ORF) 2 and 3 encoding specific HEV epitopes. We report here the development of an ELISA test to detect IgM and IgG antibodies to HEV based on these epitopes. Also contained within ORF2, is a broadly reactive epitope region (SG3) that has been useful in identifying previously undetected antibodies to HEV. In developing countries, serologic tests suggest that 1%–2% of the population have been exposed to the virus. In countries where HEV is endemic, 7%–17% of the population have measurable antibodies to HEV. In cases of acute hepatitis E, IgM and IgG antibody responses have been confirmed by PCR amplification of the virus in the sera.

Key words: Hepatitis E—Serology—Global distribution—HEV-PCR

Introduction

Hepatitis E virus (HEV) is the major etiologic agent causing enterically-transmitted non-A, non-B hepatitis (ET-NANBH) [1,2]. Epidemics of ET-NANBH have been reported in Pakistan, Burma, the former Soviet Union, India, Africa, and Mexico [1,2]. Hepatitis E is transmitted by the fecal-oral route and can be rapidly spread by contaminated water. HEV is implicated in causing more than 50% of sporadic acute viral hepatitis cases worldwide. Acute outbreaks are often associated with a mortality rate as high as 20% in infected pregnant women [1].

Diagnosis of HEV infection has been based on detection by immune electron microscopy (IEM) of virus-like particles (VLPs) in fecal specimens from persons diagnosed with acute viral hepatitis in the absence of serologic response to hepatitis A (HAV), B (HBV), C (HCV), and D (HDV) virus, Epstein-Barr virus (EBV) and Cytomegalovirus (CMV) [2]. However, IEM is difficult to perform and most clinical specimens do not contain sufficient VLPs to be detected. Until recently, the absence of a convenient and sensitive serologic test for hepatitis E hampered specific diagnosis and large-scale epidemiology studies of ET-NANBH.

The virus is a polyadenylated, positive-stranded RNA virus with three major open reading frames (ORFs) [3,4]. ORF1 is believed to encode the viral nonstructural proteins including the RNA-dependent RNA polymerase. ORF2 is believed to encode the putative capsid structural gene, while the significance of ORF3 is as yet undetermined.

Immunoreactive epitopes have been identified in both ORF2 and ORF3 [5]. The isolation of immunodominant epitopes from both the HEV Mexico and Burma strains [5] suggested that these epitopes would have utility in the diagnosis of HEV infection. These viral protein-encoding regions were subsequently used to develop an ELISA diagnostic assay to detect antibody to HEV (anti-HEV). The ELISA methodology reported here detects both IgG and IgM specific anti-HEV and is therefore useful in diagnosing acute and past HEV infection. The ELISA has been tested for possible cross-reactions to antibodies specific for other known hepatitis viruses and shown to be specific for HEV.

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Table 1. Sensitivity of the HEV enzyme-linked immunosorbent assay (ELISA).

Assay	No. of antigens	No. of samples (% of total positives) IgG	IgM
Korean panel (Kim et al)			
ORF3	2	29 (69%)	NT
ORF2/3	3	30 (71%)	NT
ORF2/3	4	42 (100%)	NT
SG3 (unique)	1	12 (29%)	NT
Pakistani/Belize panel (Ticehurst et al.)			
ORF3	2	29 (40%)	0 (0%)
ORF2/3	3	43 (59%)	2 (10%)
ORF2/3	4	73 (100%)	20 (100%)
SG3 (unique)	1	30 (41%)	18 (90%)
Saudi panel (Purcell et al)			
ORF3	2	40 (73%)	3 (9%)
ORF2/3	3	47 (87%)	24 (69%)
ORF2/3	4	55 (100%)	35 (100%)
SG3 (unique)	1	7 (13%)	11 (31%)

Each of the panels of sera were tested for antibodies to each of the four HEV recombinant antigens as described in 'Materials and Methods'. The open reading frame (ORF)3 assay includes 4-2 M and B, and 3-2 B. The ORF2/3 four antigen assay includes 4-2 M and B, 3-2 B, and SG3. Specimens with OD values greater than 0.50 with at least one HEV antigen were considered reactive. The table shows the total number of samples positive in each assay and the corresponding percentage of the total number of samples reactive with at least one antigen. Also shown are the number of samples uniquely reactive with the SG3 antigen. Data presented are previously unpublished results

U.S. and from areas in the Middle East where HEV is endemic. Twenty-nine serum samples from acute hepatitis A cases were tested for IgG and IgM antibodies to HEV. Only 1/23 of the Middle East samples was IgG positive for HEV antibody. None of the other acute hepatitis A samples were IgG- or IgM-positive for antibody to HEV. For hepatitis B, 100 samples of acute cases from the Middle East were assayed by HEV ELISA. Although 29 samples had measurable IgG antibody to HEV, only 2 were confirmed by the IgM assay. None of the 10 cases from the U.S. had detectable HEV antibody. Although 13/33 hepatitis C cases from the Middle East had detectable IgG HEV antibody, no samples tested IgM positive. For 15 serum specimens of cases of hepatitis C infection in the U.S., none reacted with the HEV antigens.

The sensitivity of the HEV ELISA is shown by comparing the number of samples that are reactive using the antigens from ORF3 and ORF2 (Table 1). The Korean panel of acute hepatitis cases showed that SG3 detected antibody in 12 (29%) additional samples that were previously undetected by the smaller 4-2 and 3-2 epitopes. The Saudi panel of 243 sporadic hepatitis cases had 55 samples that were IgG-positive to a least one HEV antigen when tested under code. All 55 cases were designated as NANB cases after serological testing for HAV and HBV. Of the 55, 7 samples (13%) were uniquely reactive with the SG3 antigen. In subsequent IgM testing, 35/55 samples were confirmed as acute HEV infections. None of the 35 specimens had detectable antibody to HCV. In the IgM assay, the SG3 antigen uniquely detected antibody to HEV in 31% of the samples. The Pakistani panel was the best indicator of the diagnostic utility of SG3. This very well-characterized panel from a point source outbreak of

Table 2. Prevalence of anti-HEV.

Samples	No. of sera	No. positive/ (% positive)	Country
Normal donor	268	9 (3.4%)	U.S./California
	168	2 (1.2%)	U.S./Texas
Normal donor	360	14 (3.9%)	Singapore
Healthy random [8]	87	3 (3.4%)	Singapore
Healthy random [9]	355	57 (16.1%)	Hong Kong
Random donor [10]	275	5 (1.8%)	Netherlands
Random donor [11]	1275	14 (1.1%)	Netherlands
Random illness [12]	100	0 (0.0%)	Germany
Healthy random [13]	1367	76 (5.5%)	Turkey
Healthy villagers [14]	374	18 (4.8%)	Sudan

The number of human sera samples with measurable IgG antibodies to HEV is shown for different global populations. Each of these panels were tested by the collaborator named with the HEV kits provided by Genelabs

HEV (Ticehurst, manuscript in preparation) demonstrates the specificity and sensitivity of the broadly reactive epitope region of SG3. Antibody to SG3 was the most frequently detected in the IgG assay. For detecting IgM anti-HEV, SG3 was by far the most sensitive antigen (90% unique positives). In a separate analysis, IgM antibody to HEV was detected within 1 month of the onset of jaundice in 62% of the cases tested (Ticehurst, personal communication). IgG antibody to HEV was detected in 94% of the cases.

Acute Hepatitis E Infection Confirmed by RT-PCR

Four serum samples from children in Giza, Egypt were assayed by HEV ELISA for IgG and IgM specific antibody. Three of the four samples were IgG-positive, one of which was confirmed by IgM testing. By RT-PCR, 2/3 antibody-positive sera were also positive for HEV-RNA. A product of the correct size that hybridized with an internal probe was observed after two rounds of PCR. This observation confirmed that HEV is endemic, and sporadic cases of HEV in children near the Kaliobiya Governorate in Egypt [7] continue to occur.

Discussion

In this study, we report the development of a prototype diagnostic test for HEV infection. The test is convenient, sensitive, and specific for anti-HEV. The HEV ELISA described here has been evaluated for its utility to detect IgG and IgM antibody to HEV and to diagnose acute HEV infections. There was little cross-reactivity for HEV when testing sera from other hepatitis disease categories. The specificity of the ELISA using recombinant antigens was further demonstrated by the observation that 425/436 (97.5%) samples from blood donors in the nonendemic U.S. were negative for antibodies to HEV (Table 2). In developing countries, serologic tests suggest that 1–2% of the population have been exposed to the virus. In countries where HEV is endemic, 7%–17% of the population have measurable antibodies to HEV.

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REPLY TO
ATTENTION OF:

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FOR THE COMMANDER:

Phylis Rinehart

Encl
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PHYLIS M. RINEHART
Deputy Chief of Staff for
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